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Evolutionary Variants of Simian Virus 40: Cloned Substituted Variants Containing Multiple Initiation Sites for DNA Replication[1]

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Two cloned evolutionary variants of simian virus 40 (SV40) containing substitutions of cellular DNA have been characterized by restriction endonuclease analysis, electron microscopic heteroduplex mapping, and DNA-DNA hybridization. Each variant genome is made up of a small, tandemly repeated segment of DNA consisting of cellular DNA and that portion of the SV40 genome containing the initiation signal for viral DNA replication. Cellular DNA sequences are different in the two variants, indicating that recombination between cell DNA and SV40-DNA can occur at more than one site. However, one end of the SV40 segment (0.68 SV40 map-units) is the same in each variant. The data suggest that substituted variants arise by integration of SV40-DNA into cellular DNA followed by excision of a small substituted genome which is subsequently amplified to a size compatible with encapsidation; the presence of multiple initiation signals in each molecule results in selective replication.

INTRODUCTION

Serial passage of SV40[2] at high multiplicity of infection leads to the evolution of new species of viral DNA containing deletions, duplications, and substitutions with cellular DNA (Uchida *et al.*, 1968; Yoshiike, 1968; Lavi and Winocour, 1972; Tai *et al.*, 1972; Brockman *et al.*, 1973; Rozenblatt *et al.*, 1973; Martin *et al.*, 1973). After many serial passages, dominant species evolve with genomes that contain DNA sequences derived largely from host-cell DNA but retain a small amount of SV40-DNA (Brockman *et al.*, 1973). Since these variant genomes replicate in cells coinfected with SV40, we postulated that the segment of SV40-DNA retained includes nucleotide sequences specifying all *cis* functions needed for viral DNA replication, including the specific initiation site.

Progress in understanding the evolution of SV40 variants has depended to a large extent on the isolation and characterization of genetically homogeneous variants, i.e., cloned populations of single variants, from both early passage and late passage stocks (Brockman and Nathans, 1974; Mertz and Berg, 1974). In the preceding paper, Brockman *et al.* (1975) described the characterization of cloned variants from early passage virus which can express some SV40 genes. In this communication we describe the properties of cloned substituted variants present in late passage stocks.

In contrast to early passage virus (Brockman and Nathans, 1974; Brockman *et al.*, 1974; Mertz *et al.*, 1974; Brockman *et al.*, 1975), the genomes of late variants are made up of short, tandemly repeating seg-

[1] This is publication No. 15 in a series on the genome of simian virus 40. Publication No. 14 is Brockman *et al.* (1975).

[2] The following abbreviations are used: SV40, simian virus 40; DNA form I, covalently closed-circular duplex DNA; DNA form II, circular duplex DNA with one or more single-strand scissions; SV40-DNA-L_{RI}, linear SV40-DNA prepared with Endo R·*Eco*RI. Abbreviations for restriction endonucleases follow the conventions described in Smith and Nathans (1973).

ments of DNA, consisting of cellular DNA and that portion of the SV40 genome which contains the initiation signal for viral DNA replication. Therefore, the initiation site is present in multiple copies, probably accounting for the predominance of these variants in late passage stocks. Since genomes with very small segments of SV40-DNA replicate in cells coinfected with helper virus, it is likely that no *cis* functions other than the initiation signal are required for SV40-DNA replication. Moreover, the two substituted variants examined in detail contained nonhomologous cellular DNA sequences, indicating that recombination between SV40-DNA and cell DNA can occur at more than one site. A preliminary account of part of this work has been published elsewhere (Brockman and Nathans, 1974; Brockman *et al.*, (1974).

MATERIALS AND METHODS

Cells and viruses. These have been described in prior publications (Danna *et al.*, 1973; Brockman and Nathans, 1974). Briefly, small-plaque SV40 (from strain 776) was grown in BSC-1 cells infected at an input multiplicity of 0.001 PFU/cell, starting with a stock made from a single plaque. Serial undiluted passage of this plaque-purified stock has been described (Brockman *et al.*, 1973); P13 and P20 are the 13th and 20th serially passed stock virus, respectively. The procedure for cloning variants from P13 and P20 has been described (Brockman and Nathans, 1974).

Viral DNA. DNA labeled with ^{32}P or with [^{14}C]thymidine was prepared from infected cells by the procedure of Hirt (1967), as detailed earlier (Danna *et al.*, 1973). Following the isolation of DNA form I by equilibrium centrifugation, in CsCl–ethidium bromide solution, the dialyzed sample was subjected to electrophoresis in 1.4% agarose gel as described previously (Brockman and Nathans, 1974). In the case of [^{32}P]DNA the variant and helper SV40-DNA were detected by autoradiography and in the case of [^{14}C]DNA by ethidium bromide staining (Sharp *et al.*, 1973). The short, variant DNA and the SV40-DNA were recovered by electrophoresis into dialysis sacs. In some cases, equilibrium centrifugation was omitted in the purification of variant DNA. ^{14}C-labeled cell DNA was prepared from BSC-1 cells as described (Brockman *et al.*, 1973).

Digestion of viral DNA. Digestion with various restriction endonucleases (Endo R·*Hin*dII + III, R·*Hin*dIII, R·*Eco*RI and R·*Eco*RII) and isolation of DNA fragments by electrophoresis in acrylamide-gel slabs have been described (Danna *et al.*, 1973). Endo R·*Hin*dII + III and R·*Hin*dIII were prepared as described earlier (Lai and Nathans, 1974); Endo R·*Eco*RI was prepared by P. Geshelin, following the procedure of Yoshimori (1971); Endo R·*Eco*RII was prepared through the ammonium sulfate step by the procedure of Yoshimori (1971) and then chromatographed on diaminobutane-substituted Sepharose (Shaltiel and Er-El, 1973) as described for R·*Hpa* I (Sack, 1974). To quantitate [^{32}P]DNA fragments in electrophoresis gels, autoradiograms were traced with a Joyce-Loebl microdensitometer and the areas of expanded peaks measured.

DNA–DNA hybridization. ^{32}P-labeled variant DNA was hybridized to ^{14}C-labeled SV40-DNA, to ^{14}C-labeled SV40-fragments, or to ^{14}C-labeled BSC-1 (cellular)-DNA, essentially as described previously (Brockman *et al.*, 1973). In some instances the DNA was nicked and denatured by heating at 100° in $1/100$ SSC for 15 min.; in other instances the DNA was boiled in 0.2 *N* NaOH for 30 min. To follow rates of reannealing of denatured ^{32}P-labeled variant or SV40-DNA, alkali-boiled DNA was incubated under oil in 0.3 *M* NaCl, 20 m*M* Tris–Cl, pH 7.6, 1 m*M* EDTA at 68°, and samples were removed periodically and chilled. The percentage of DNA remaining single stranded at various times was determined by adsorption of double-stranded DNA onto hydroxyapatite at 65° in 0.14 *M* sodium phosphate, pH 6.8–0.15 *M* NaCl and counting an aliquot of the supernatant fraction (Brockman *et al.*, 1973). In some experiments S1 nuclease (Sutton, 1971) was used to assess single-stranded DNA (Brockman *et al.*, 1973).

Electron microscopy of DNA. For length

measurements of variant DNA, variant form II DNA isolated by electrophoresis in agarose was mixed with SV40-DNA-L_{RI} and mounted on grids as described by Davis *et al.* (1971). For heteroduplex mapping, an Endo R fragment of variant DNA was mixed with SV40-DNA-L_{RI} (1 μg/ml) at a molar ratio of 10–20 (fragment to L_{RI}), and the mixture denatured, renatured, and mounted according to the procedure of Davis *et al.* (1971). Heteroduplexes between DNA fragments were prepared in a similar way, as described in the text, except that the molar ratio was about 1:1. To measure DNA lengths, projections of electron micrographs were traced with a Graphics calculator (Numonics Corp.).

Replicative intermediates of ev-1101. Replicative intermediates, pulse-labeled with [^{3}H]thymidine as described elsewhere (Brockman *et al.*, 1975), were separated from cellular DNA by the method of Hirt (1967) followed by treatment with heated RNase, phenol extraction and alcohol precipitation. The viral DNA was then digested to completion with Endo R·*Hpa* I to cleave wild type helper DNA (Sack and Nathans, 1973) and centrifuged through a 5–30% sucrose gradient at 40,000 rpm and 10° for 7 hr in a Spinco SW41 rotor. Fractions containing radioactive DNA (about 24 S) were pooled and further processed as described in the text.

RESULTS

Isolation of Cloned Variants from Late Passage Stocks

To isolate cloned variants of SV40, which have retained only a small portion of the SV40 genome, we used virus stocks from the 13th and 20th serial passages (P13 and P20). Cells infected with either of these stocks yield viral DNA consisting of a few dominant and abnormal species with mostly cellular DNA sequences (Brockman *et al.*, 1973). In the cloning procedure, P13 or P20 lysate (containing defective and helper viruses) was plated on BSC-1 cells at various dilutions, and individual plaques were screened for the presence of variant genomes, as described by Brockman and Nathans (1974). Viral DNA prepared from cells infected with plaqued virus stocks were surveyed in two ways: 1) By agarose-gel electrophoresis to detect short genomes, and 2) by digestion with Endo R·*Hin*dII + III to detect new DNA fragments expected from a variant genome. Of 34 plaqued stocks from P13, four had evidence for short genomes, and two of five plaqued stocks from P20 had variants, which in this case were identical to each other. Of the five variants detected, we chose three for further characterization: *ev*-1101 and -1103 isolated from P13, and *ev*-1102 isolated from P20 (Fig. 1).

Length of Variant DNA's

Variant genomes isolated from infected cells and separated from helper genomes by agarose-gel electrophoresis were measured in the electron microscope. The results, presented in Fig. 2, indicate that *ev*-1101 was 85 ± 5% (SD) of the length of SV40-DNA present on the same grid, *ev*-1102 was 79 ± 5%, and *ev*-1103 was 78 ± 4%. As seen in Fig. 1, however, *ev*-1103 genomes were not uniform in length, the above measurements being done on the dominant species. As will be indicated below, the different size-classes of DNA derived from this variant are related, since they yielded the same Endo R·*Hin*d fragments.

Endo R Digests of Variant DNA's

As already noted, SV40 variants from P13 and P20 virus were detected by the Endo R·*Hin*d digest pattern of DNA containing variant plus helper virus genomes. For a clearer analysis, short genomes isolated by agarose-gel electrophoresis were digested and the resulting fragments subjected to electrophoresis. Typical results are shown in Fig. 3. As seen in the figure, *ev*-1101 DNA yields a single *Hin* fragment which is 17% of the length of SV40-DNA, as determined by mobility (Danna *et al.*, 1973); *ev*-1102 DNA yields two *Hin* fragments which are 20 and 7%, respectively, of SV40-DNA; and *ev*-1103 yields two fragments, which are 4.6 and 4.2%, respectively, of SV40-DNA. These results indicate that each of these DNA's consists of tandemly repeated segments. For further

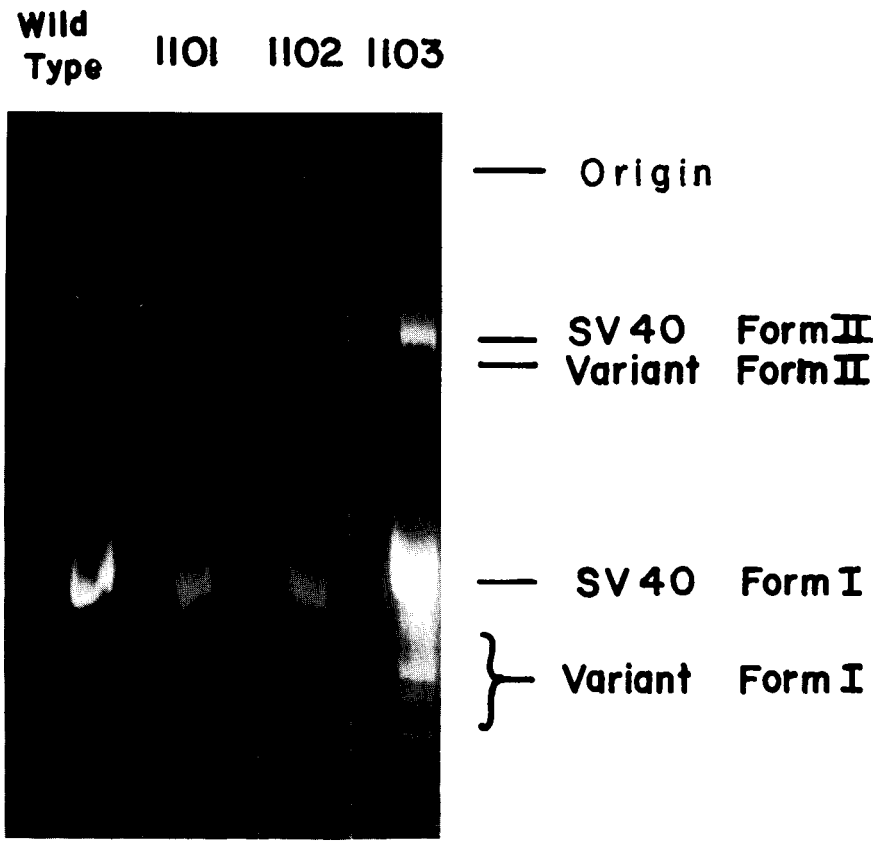

FIG. 1. Separation of short, variant DNA molecules from SV40-DNA by electrophoresis in agarose: ethidium bromide stain of viral DNA purified as described in Materials and Methods.

characterization, we concentrated on *ev*-1101 and *ev*-1103, the two simpler variants.

Since *ev*-1103 yielded two *Hin* fragments, we could not be certain that these were derived from the same variant DNA, even though the two fragments were produced in equimolar amounts (Table 1). Therefore, we looked for a restriction endonuclease which yielded only one fragment from *ev*-1103 DNA. Endo R·*Eco*RII yielded a single fragment which was 8.8% of the length of SV40-DNA as judged by electrophoretic mobility (Fig. 4). This fragment as well as partial RII digest products (17.5 and 26% of unit length; see Fig. 4) were redigested with R·*Hin*dIII to yield the products also shown in Fig. 4. The amount of each fragment was then estimated from densitometer tracings and molar yields calculated (Table 1). From these results we conclude that *ev*-1103 DNA has a repeating unit of about 8.8% of the length of SV40-DNA, each unit having two *Hin*d sites and one *Eco*RII site, and that the RII site is within the *Hin*-I_1 segment of the molecule (Fig. 5). Also, the results presented in Table 1 confirm the presence of tandemly repeated segments in *ev*-1103 DNA.

As pointed out earlier, *ev*-1103 DNA isolated from infected cells consists of more than one size-class, as indicated by agarose-gel electrophoresis of the open-circular form (Fig. 1). When each of the minor size-classes was isolated from the gel and analyzed by Endo R·*Hin*d digestion, the electrophoretic pattern of fragments was the same in all cases and corresponded to that shown in Fig. 3. Since *ev*-1103 DNA has a repeating unit of about 8.8% of

SV40-DNA length, the shorter molecules probably lack one or more repeating units relative to the major size-class.

Rates of Reassociation of Denatured Variant DNA

As a further test of the complexity of variant DNA's, we compared the rates of reassociation of denatured variant DNA to that of helper SV40-DNA isolated from the same infected cells. If variant DNA is made up of *n* repetitious segments of identical sequence per SV40-DNA equivalent, the rate constant for renaturation should be *n* times that of SV40-DNA. In order to compare variant and SV40-DNA as precisely as possible, ^{32}P-labeled variant and helper virus DNA's were isolated from the same batch of infected cells by agarose-gel electrophoresis of form I DNA (see Materials and Methods). (In each case the isolated helper virus DNA yielded a wild-type *Hin* digest pattern.) Each DNA sample was then boiled in alkali to fragment the DNA, and the rates of renaturation were measured as described, using hydroxyapatite to adsorb duplex DNA.

The results are shown in Fig. 6 as a plot of the reciprocal of the fraction remaining single-stranded (1/fss) vs the product of initial DNA concentration (cpm/ml) and time at 68° (C_0t) divided by the product of initial DNA concentration and time for 50% annealing of the reference SV40-DNA ($C_0t_{1/2}$ (SV40)). From the slope of each line we have estimated the relative rate constant for *ev*-1101 DNA as five times that of SV40-DNA and the rate constant for *ev*-1103 DNA as 10–12 times that of SV40-DNA. These values agree well with the results of Endo R·*Hin* digestion, i.e., *ev*-1101 DNA has a repetitious segment of DNA which is slightly more than ⅙ of the length of SV40-DNA, and *ev*-1103 DNA has a repetitious segment which is about 1/11 of the length of SV40-DNA. Therefore, both sets of data indicate that each variant genome is made up of identical or nearly identical repetitious segments of DNA.

The Presence of SV40 and Cell DNA Sequences

As pointed out earlier, P13 and P20 were known to contain predominantly nonrepetitive cell DNA and relatively little SV40-DNA or reiterated cell DNA (Brock-

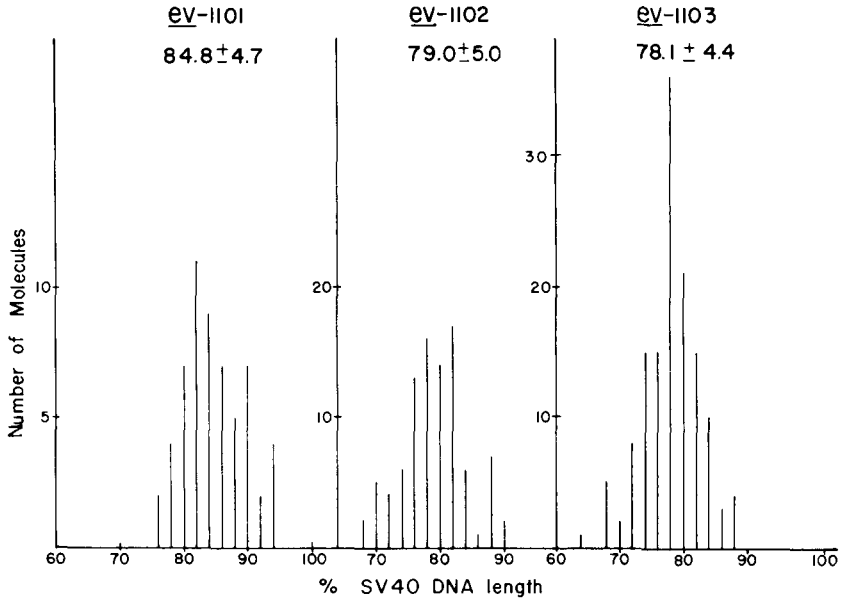

FIG. 2. Lengths of variant DNA's compared to SV40-DNA: Electron microscopic measurements. Form II variant DNA's were isolated by electrophoresis in agarose, mixed with SV40-DNA-L_{RI} and mounted for microscopy.

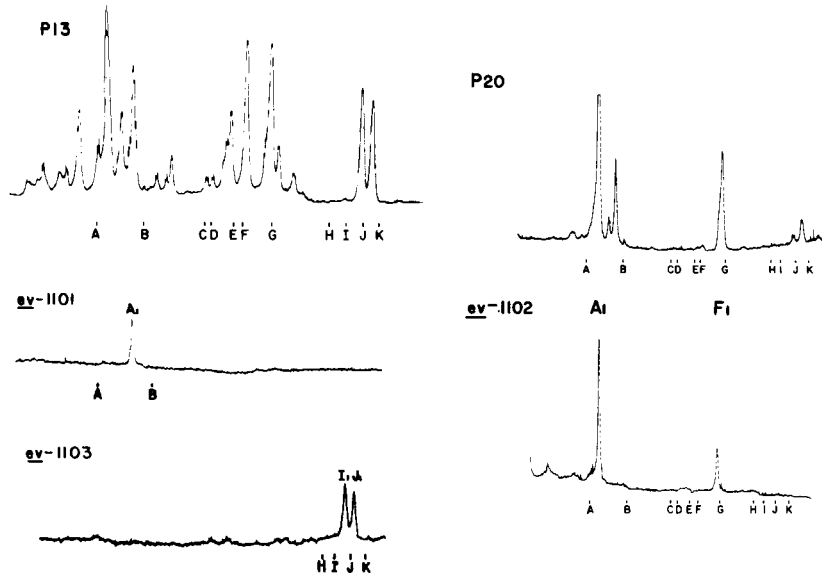

FIG. 3. Endo R·*Hin* digests of variant DNA's: Tracing of autoradiograms of slab gels after electrophoresis. On the left is the digest pattern of P13-DNA, of *ev*-1101 DNA isolated by electrophoresis in agarose, and of *ev*-1103 DNA isolated by electrophoresis in agarose. On the right is the digest pattern of P20-DNA and of *ev*-1102 DNA isolated by electrophoresis in agarose. In each case the origin is at the left, and A,B,C ... K represent the positions of SV40 *Hin* fragments in the same gel.

TABLE 1

PRODUCTS OF *Hind* AND *Eco*RII DIGESTION OF *ev*-1103 DNA

Digested DNA	Enzyme	Fragments (% of SV40-DNA)	Relative yield [% cpm (moles)]
ev-1103	*Hind*II + III	4.6 (I1)	51 (1)
	or *Hind*III	4.2 (J1)	49 (0.96)
ev-1103	*Eco*RII	8.8 (monomer)	—
		17.5 (dimer)	—
		26.0 (trimer)	—
*Eco*RII monomer	*Hind*III	4.2 (J1)	50 (1)
		3.1 (I1a)	34 (0.89)
		1.5 (I1b)	16 (0.90)
*Eco*RII dimer	*Hind*III	4.6 (I1)	27 (1)
		4.2 (J1)	47 (1.90)
		3.1 (I1a)	17 (0.92)
		1.5 (I1b)	8.6 (0.97)
*Eco*RII trimer	*Hind*III	4.6 (I1)	34 (2.0)
		4.2 (J1)	48 (3.1)
		3.1 (I1a)	11.5 (0.98)
		1.5 (I1b)	6.5 (1.2)

man *et al.*, 1973). We now wished to analyze the DNA of cloned variants for the types of sequences present. For this purpose we incubated low concentrations of denatured ^{32}P-labeled variant DNA with excess denatured ^{14}C-labeled SV40-DNA or ^{14}C-labeled BSC-1-DNA, as described in Materials and Methods, and measured the

percentage of [^{32}P]DNA remaining single stranded after 1 hr at 68°. (BSC-1-DNA was used under conditions where only repetitive sequences renatured, i.e., at C_0t of 0.3 mole sec/liter.) The percent of variant DNA hybridized was assessed in two ways. In one procedure, heat-nicked and -denatured DNA was used, and hybridization was assessed by hydroxyapatite adsorption of duplex DNA, as detailed in Materials and Methods. This procedure detects even small amounts of SV40 or reiterated host DNA, since the heat-nicked DNA varied from ⅓ to full length (Brockman *et al.*, 1973), and partial duplexes adsorb to hydroxyapatite. In the second procedure DNA was denatured and broken by boiling in alkali, and duplexes were assessed by digestion with the single-strand-specific nuclease S1 (Ando, 1966). This method measures the percentage of sequences derived from SV40 or reiterated host DNA.

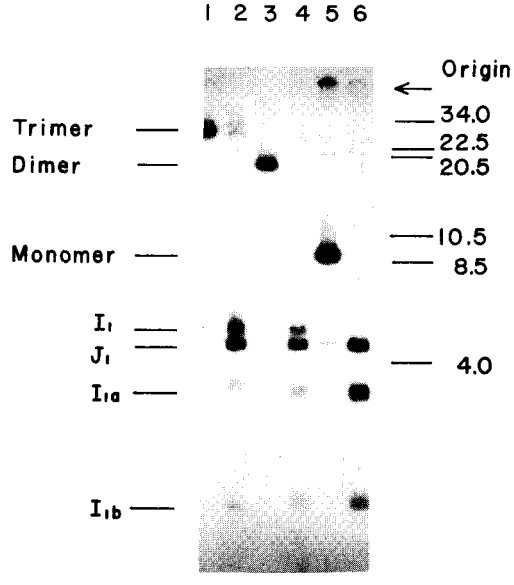

FIG. 4. *Hin*dIII digests of *Eco*RII fragments of *ev*-1103 DNA: Autoradiograms of acrylamide gels after electrophoresis. Column 1, *Eco*RII trimer; column 2, *Hin*dIII digest of trimer; column 3, *Eco*RII dimer; column 4, *Hin*dIII digest of dimer; column 5, *Eco*RII monomer; column 6, *Hin*dIII digest of monomer. I1, J1, I1a and I1b are digest products as noted in the text and Table 1. On the right are the positions of *Hin*dIII fragments of SV40-DNA and their respective lengths, as percent of SV40-DNA.

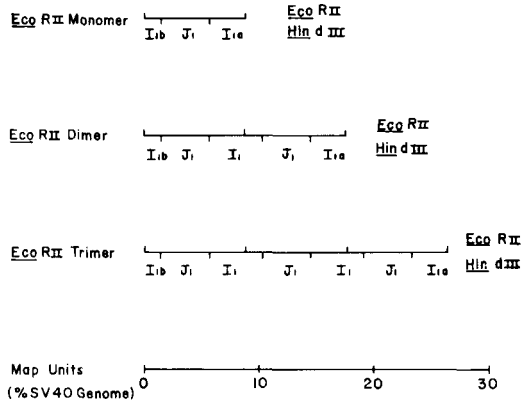

FIG. 5. Diagram of *Hin* and *Eco*RII sites in *ev*-1103 DNA, deduced from the data shown in Figs. 3 and 4 and Table 1 (see text).

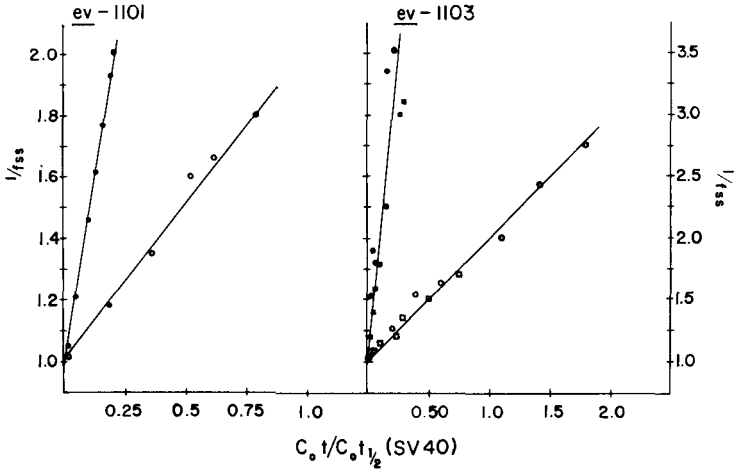

FIG. 6. Rates of reannealing of variants DNA's compared to SV40-DNA. fss and $C_0t/C_0t_{1/2}$ (SV40) are defined in the text, and are plotted following Wetmur and Davidson (1968).

The results are presented in Table 2.

As shown in the table (heat, HA) *ev*-1101 DNA contains SV40 sequences but no reiterated BSC-1 sequences; *ev*-1103 contains both. In the case of *ev*-1101, 47% of the DNA hybridized to SV40-DNA and <5% to repetitive cellular DNA (Table 2, alkali, S1). Since only 79% of the ^{14}C-labeled SV40-DNA in the same tube hybridized under these conditions, we estimate that about 59% (47/79) of *ev*-1101 DNA corresponds to SV40-DNA sequences, and <5% corresponds to reiterated cell DNA; the remainder presumably was derived from nonreiterated or less reiterated cell DNA. In the case of *ev*-1103, about 20% of the DNA was derived from SV40-DNA, about 15% from reiterated cell DNA, and about 65% presumably from nonreiterated or less reiterated cell DNA.

Since *ev*-1101 DNA yielded two *Hin* fragments, similar experiments were done with the individual fragments of this variant DNA. As shown in Table 2, *Hin*-I_1 contained both SV40 and reiterated cell sequences and *Hin*-J_1 contained little or no SV40 or reiterated cell sequences. Therefore, J_1 consists largely or entirely of nonreiterated or less reiterated cell DNA.

The Presence of Specific SV40-DNA Sequences

Having demonstrated the presence of SV40 sequences in *ev*-1101 and *ev*-1103 DNA's, we next determined which regions of the SV40 genome were retained. For this purpose denatured ^{32}P-labeled variant DNA was incubated with an excess of each SV40 *Hin* fragment, one at a time and labeled with ^{14}C (see Materials and Methods). The percentage of [^{32}P]DNA remaining single stranded was then determined after 1 hr at 68° by the hydroxyapatite method. The results are presented in Table 3.

In the case of *ev*-1101, contiguous *Hin* fragments A and C hybridized to variant DNA, whereas in the case of *ev*-1103 only *Hin*-C hybridized. In some experiments there was slight hybridization of *Hin*-D to *ev*-1101 DNA (Table 3); this was probably due to the demonstrable contamination of this *Hin*-D preparation with *Hin*-C, since other preparations of *Hin*-D showed <5% hybridization. We conclude that *ev*-1101 DNA contains a segment of SV40-DNA derived from the *Hin*-AC region of the DNA, whereas *ev*-1103 DNA contains a segment of SV40-DNA derived from the *Hin*-C region. From these experiments, however, we cannot exclude the presence of other SV40-DNA segments which might be too short to result in binding to hydroxyapatite. Indeed, nucleotide sequence analyses of *ev*-1103 DNA indicate that a small part of *Hin*-A is present (Dhar and Weissman, personal communication).

Heteroduplex Mapping of SV40 Sequences in Variant DNA's

To confirm the results of hybridization of variant DNA with *Hin* fragments and to map the SV40 segments more precisely, we have examined by electron microscopy heteroduplex molecules consisting of a linear strand of SV40-DNA made with Endo R·*Eco*RI (SV40-DNA-L_{RI}) and an Endo R fragment from *ev*-1101 or *ev*-1103 DNA. In the case of *ev*-1101 the fragment was the single *Hin* fragment present in an *Hind* digest (Fig. 3) which is 17% of the length of SV40-DNA. In the case of *ev*-1103, we used the *Eco*RII cleavage products which were 8.8% of the length of SV40-DNA or multimers of this segment (Table 1). The results are shown in Figs. 7 and 8. In the figures are micrographs of representative heteroduplex molecules and length measurements of individual molecules.

As seen in the micrograph of an *ev*-1101/SV40 heteroduplex (Fig. 7), the *ev*-1101 fragment has cyclized on the SV40-DNA strand. This is the result expected if the *Hin* cleavage site is within the SV40 sequence of the variant. The *ev*-1101 fragment has paired with single-strands of SV40-DNA-L_{RI} at a unique site beginning at 55.7 ± 4.5% of the length of SV40-DNA from one end and 32.3 ± 4.7% from the other end (Fig. 7). Since *ev*-1101 DNA has *Hin*-A and -C sequences, we can orient the SV40-DNA strand with the *Hind* cleavage map (Fig. 7, bottom right). From the measured lengths of different parts of heteroduplex molecules of this type, we conclude that the duplex region extends from

TABLE 2

HYBRIDIZATION OF VARIANT DNA TO SV40-DNA OR BSC-1-DNA[a]

Variant DNA	Method	Percent hybridized			Percent SV40 sequences	Percent reiterated BSC sequences
		No added DNA	+ SV40-DNA	+ BSC-1-DNA		
ev-1101	Heat, HA	12	64 (91)	0 (29)		
ev-1101	Alkali, S1	0	47 (79)	0 (16)	59	<5
ev-1103	Heat, HA	18	62 (84)	68 (47)		
ev-1103	Alkali, S1	9	18 (90)	15 (21)	20	15
ev-1103 I1	Alkali, S1	0	38 (86)	23 (13)	44	23
ev-1103 J1	Alkali, S1	0	3 (87)	3 (14)	<5	<5

[a] A mixture of ^{32}P-labeled variant DNA and ^{14}C-labeled SV40-DNA (or ^{14}C-labeled BSC-1-DNA) was denatured as indicated under Materials and Methods (100° in 1.5 m*M* NaCl or boiling in alkali) and incubated at 68° for 1 hr. Percent hybridized is percent of ^{32}P removed by hydroxyapatite (HA) or resistant to S1 nuclease. Figures in parenthesis are percent [^{14}C]DNA hybridized in the same tube. Percent SV40 sequences = (% variant DNA hybridized in S1 experiment/% SV40-DNA hybridized in same tube) × 100. Percent reiterated BSC sequences = percent variant DNA hybridized in presence of BSC-1-DNA, using S1 nuclease. 290 cpm of *ev*-1101 DNA was used (7 ng/ml); 300–400 cpm of *ev*-1103 DNA (4–8 ng/ml); and 100 cpm of I1- or J1-DNA (2–5 ng/ml).

TABLE 3

HYBRIDIZATION OF VARIANT DNA TO SV40 *Hin* FRAGMENTS[a]

Hin fragment	Percent variant DNA hybridized	
	ev-1101	*ev*-1103
A	76	0
B	9	1
C	59	32
D	18 (0)	5
E	7	0
F	0	0
G	3	0
H	0	0
I	1	0
J	6	0
K	0	0

[a] A mixture of heat-denatured ^{32}P-labeled variant DNA (240 cpm for 1101 and 230 cts/min for 1103) and excess ^{14}C-labeled *Hin* fragment were incubated at 68° in 0.3 *M* NaCl; duplex DNA was then adsorbed by hydroxyapatite (Brockman *et al.*, 1973). The percent variant DNA hybridized is the percent of ^{32}P removed by hydroxyapatite, taking as 100% the ^{32}P unadsorbed in the absence of added fragment (82% of input 1101 DNA and 83% of input 1103 DNA). In each experiment ≥90% of each ^{14}C-labeled *Hin* fragment reannealed. A second experiment with *ev*-1101 DNA and a different D fragment preparation gave the value indicated in the parentheses.

0.56–0.68 map-units in the SV40 cleavage map. Therefore, the *ev*-1101 *Hin* fragment has *Hin*-C sequences at one end and *Hin*-A sequences at the other end; cleavage by Endo R·*Hin*d occurred at the *Hin*-A·C junction (Fig. 7). (As reported earlier (Danna *et al.*, 1973), this site is an Endo R·*Hin*dIII site, and, as expected, *ev*-1101 DNA is cleaved to the 17% fragment by purified Endo R·*Hin*dIII.) From measurements of the duplex region we estimate that about 65% of the sequences present in *ev*-1101 DNA are derived from SV40-DNA; this is close to the estimate of 59% SV40-DNA sequences based on extent of hybridization with SV40-DNA (Table 2).

The *ev*-1103 *Eco*RII fragments also pair with single strands of SV40-DNA-L_{RI} at a unique site (Fig. 8). This site is at about 65–68% from one end of the SV40-DNA, which is consistent with sequences around the *Hin*-A·C junction (Fig. 8), as predicted from the results already presented. From the measurements of heteroduplex molecules, we estimate that about 34% of the sequences present in *ev*-1103 DNA are derived from SV40-DNA; this compares with the estimate of 20% based on extent of hybridization to SV40-DNA (Table 2).

Comparison of Cell DNA Sequences in ev-1101 and *ev*-1103

It was of interest to determine whether the cell DNA sequences present in *ev*-1101 and *ev*-1103 are related, since they might have arisen from a common ancestral mol-

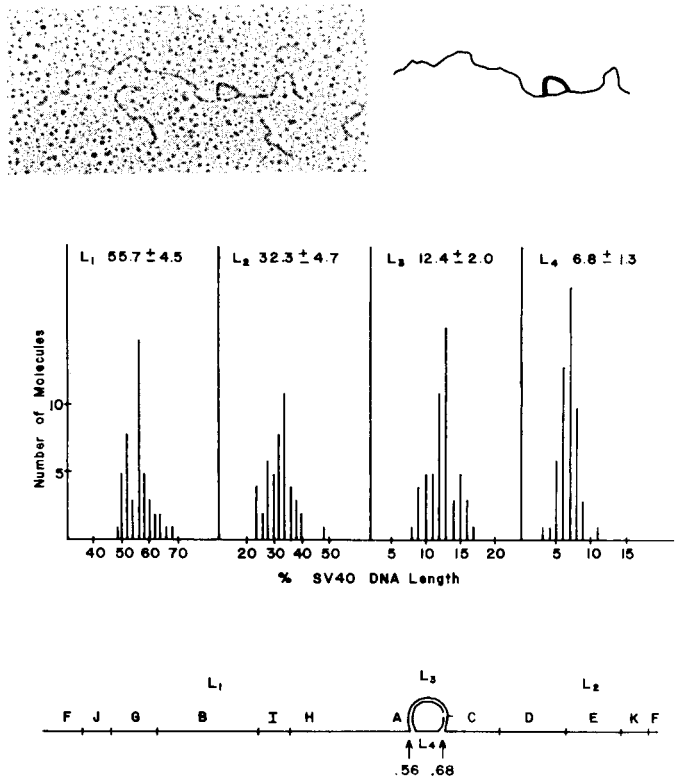

FIG. 7. Analysis of SV40-DNA-L_{RI}/*ev*-1101 *Hin* fragment heteroduplexes. Shown are a representative heteroduplex molecule (upper left); a tracing of the molecule (upper right); length measurements on a series of such molecules (middle); and a model (bottom) indicating *Hin* cleavage sites, lengths measured, and map units of the duplex region (taken from the graphs of L1 and L2).

ecule or they could have been generated by independent recombinations between SV40-DNA and cell DNA at different or identical sites. From the results already presented it is clear that the cell DNA sequences in the two variants are not identical, since *ev*-1103 has both reiterated and nonreiterated cell sequences whereas *ev*-1101 has no reiterated sequences (Table 2). For further comparison the *Hin*-I_1 and -J_1 fragments of *ev*-1103 DNA were hybridized to *ev*-1101 DNA. As seen in Table 4, about the same percentage of *Hin*-I_1 sequences hybridized to *ev*-1101 DNA as to SV40-DNA. Since *ev*-1101 DNA appears to contain all detectable SV40 sequences present in *ev*-1103 DNA (see also Figs. 7 and 8), within the limits of error of the hybridization measurements, *Hin*-I_1 DNA and *ev*-1101 DNA share only SV40 sequences. In the case of *Hin*-J_1, neither *ev*-1101 nor SV40-DNA hybridized with this fragment. Therefore, *Hin*J_1 and *ev*-1101 DNA do not share sequences detectable by this method. We conclude that most or all of the host sequences present in one variant differ from those in the other.

A similar conclusion arises from examining heteroduplex molecules consisting of one strand of *ev*-1101 *Hin* fragment and one strand of *ev*-1103 *Eco*RII fragment (Fig. 9). Although length measurements are imprecise with such small fragments and heteroduplex molecules were rare, the heteroduplex structures seen are consistent with duplex formation only at the shared SV40 sequences around the *Hin*-A·C junction (Fig. 9).

Origin of DNA Replication in Variant DNA

Although every variant we have examined to date retains the region of SV40-

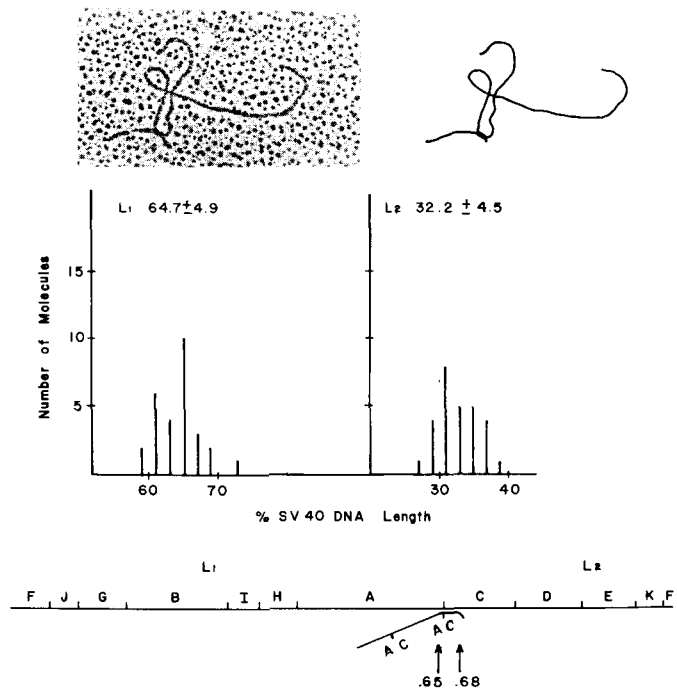

FIG. 8. Analysis of SV40-DNA-L_{RI}/*ev*-1103 *Eco*RII fragment heteroduplexes. Shown are a representative heteroduplex molecule (upper left); a tracing of the molecule (upper right); length measurements (middle); and a model (bottom) indicating *Hin* cleavage sites and map units of the duplex region (taken from the graphs).

TABLE 4

HYBRIDIZATION OF *ev*-1103 *Hin* FRAGMENTS TO SV40 OR *ev*-1101 DNA[a]

ev-1103 Fragment	DNA in excess	Concentration (μg/ml)	Percent fragment hybridized	Percent SV40 or *ev*-1101 sequences
I1	SV40	2.9	31 (80)	39
I1	SV40	5.8	35 (84)	42
I1	*ev*-1101	1.6	35 (82)	43
I1	*ev*-1101	3.2	30 (87)	34
J1	SV40	2.9	<5 (80)	<5
J1	SV40	5.8	<5 (84)	<5
J1	*ev*-1101	1.6	<5 (83)	<5
J1	*ev*-1101	3.2	<5 (87)	<5

[a] Approximately 115 cpm of ^{32}P fragment (1 ng) was mixed with ^{3}H-labeled SV40 or ^{3}H-labeled *ev*-1101 DNA and the mixture incubated as detailed in Materials and Methods. The mixture was then treated with S1 nuclease and TCA-precipitable and -soluble radioactivity determined. In the absence of added DNA, 22 and 18% of I1- and J1-DNA, respectively, was S1-resistant. Percent fragment hybridized is the percent of input ^{32}P in the TCA precipitate, correcting for the control just indicated. Nearly identical results were obtained using either TCA-soluble or precipitable counts. The values in parentheses are the percent [^{3}H]DNA which was S1 resistant in each tube; these values were used to normalize percent fragment hybridized, as indicated in the last column.

DNA which contains the origin of DNA replication (Danna and Nathans, 1972; Fareed *et al.*, 1972), we wished to demonstrate more directly that a substituted variant does begin replication at the SV40 signal and not within its cell DNA segment. To determine the position of replication initiation in *ev*-1101, this site was

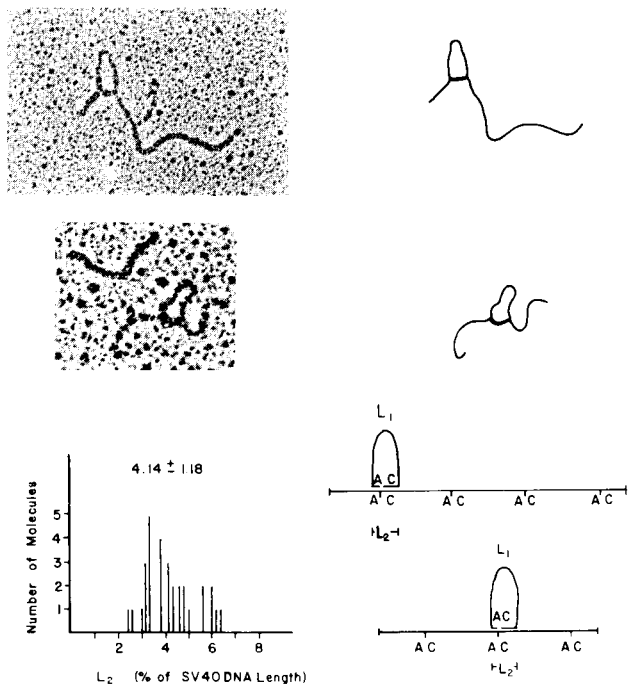

FIG. 9. Analysis of *ev*-1101 *Hin* fragment/*ev*-1103 *Eco*RII fragment heteroduplexes. Shown are two representative heteroduplex molecules (upper left); a tracing of the molecules (upper right); measurements of several molecules of this type (lower left); and diagrams indicating *Hin* cleavage sites. The loop is derived from the *ev*-1101 fragment.

located relative to the nearest *Hin*dIII cleavage site. For this purpose replicating molecules of *ev*-1101 were partially cleaved with Endo R·*Hin*dIII, which cleaves at the *Hin*-A·C junction. Since the length of the *ev*-1101 repeating unit is known, each A·C junction can be located along the cleaved molecule and the initiation site (taken as the midpoint of a replicating branch; (Schnös and Inman (1970); Fareed *et al.* (1972)) can be localized with respect to this junction. The stocks of *ev*-1101 used to prepare replicating intermediates, however, contain wild-type virus in addition to the variant. Because the partial digest products of wild-type replicative intermediates would make the interpretation of the digestion results difficult, wild-type replicative intermediates were removed prior to *Hin*dIII digestion. This was accomplished by digesting to completion the pulse-labeled DNA preparation containing *ev*-1101 and wild-type replicative intermediates with Endo R·*Hpa*I. This enzyme cleaves wild-type SV40 into three fragments (Sack and Nathans, 1973; Sharp *et al.*, 1973) but does not cleave *ev*-1101 DNA. When the *Hpa*I-digested DNA was subjected to velocity centrifugation through a 5–20% sucrose gradient, the wild-type fragments were readily separated from replicating and mature molecules of *ev*-1101. The broad peak of *ev*-1101 replicative intermediates sedimenting at about 24 S was isolated and partially digested with *Hin*dIII so that the majority of cleaved molecules contained only one scission. The distance of the replication initiation site from the *Hin*dIII site in full-length *ev*-1101 replicating molecules was measured in the electron microscope (Fig. 10) and was expressed in terms of the repeating unit, using the formulae given in the legend to Fig. 10. As shown in the figure, the majority of *ev*-1101 molecules analyzed started replication at a site close to a *Hin*dIII

cleavage site. (Although the orientation of molecules visualized is not known, since the origin of replication in most molecules is very close to the A·C junction, the orientation has little effect on the length distributions.) The initiation point thus falls at or near the *Hin*-A·C junction, which corresponds to the origin of wild-type SV40-DNA replication (Danna and Nathans, 1972).

Since *ev*-1101 DNA contains five possible initiation sites for DNA replication, we examined replicating molecules for multiple replication loops. Of 30 replicating molecules measured, ten were <50% replicated. None of these molecules (nor those >50% replicated) had more than one replication loop.

DISCUSSION

In Fig. 11 we summarize our results on the structure of *ev*-1101 and *ev*-1103 DNA in relation to the *Hin* cleavage map of the SV40 genome. As shown in the figure, *ev*-1101 DNA is made up of a repeating segment which is 17% of the length of

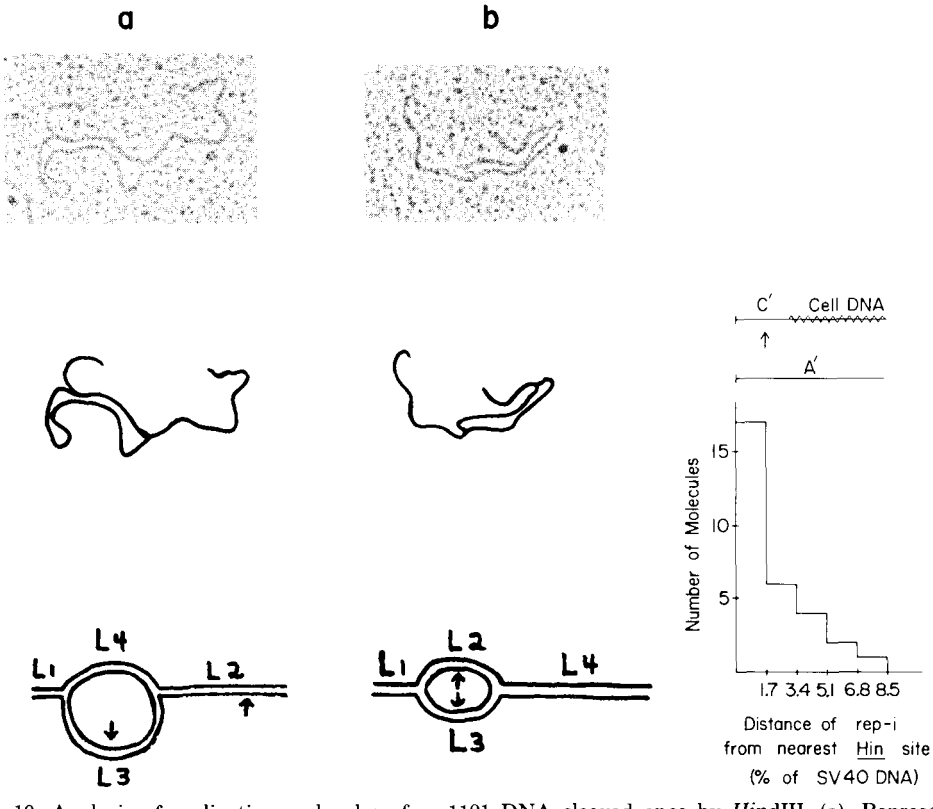

FIG. 10. Analysis of replicating molecules of *ev*-1101 DNA cleaved once by *Hin*dIII. (a), Representative molecule that has been cleaved in one of the replicated branches. In molecules of this type the unbroken circle was equal to the length of *ev*-1101 genomes present on the same grid. (b), Representative molecule that has been cleaved in an unreplicated branch. In molecules of this type the circle varied from a small fraction of the length of *ev*-1101 DNA to nearly twice the length of variant DNA. The arrows in the models indicate the midpoint of each replication loop, taken as the origin of replication. The distance of the initiation site (rep-i) from a *Hin*dIII site, expressed as a multiple of *ev*-1101 DNA *Hin* fragment, was computed as follows: For molecules of type (a), $5\times[(L1 + L2)/2 + L1 + L4]/(L1 + L4 + L2)$; for molecules of type (b), $5\times[L1 + (L2 + L3)/4]/[L1 + (L2 + L3)/2 + L4]$. For the histogram shown on the right, the distance of rep-i from the *nearest Hin*dIII site (*Hin*-A·C junction) is expressed as percent of SV40-DNA length, taking the distance between A·C junctions as 17% of the length of SV40-DNA. At the top of the graph are shown the two possible orientations of *ev*-1101 DNA near the *Hin* cleavage site.

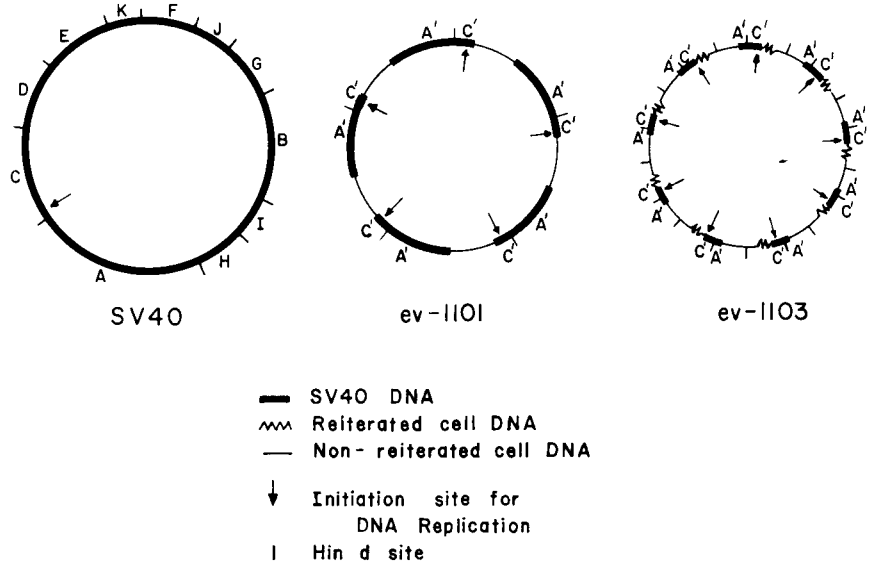

FIG. 11. The structure of the genomes of *ev*-1101 and *ev*-1103, compared to the *Hin* cleavage map of SV40 (Danna *et al.*, 1973).

SV40-DNA. This segment is repeated five times in the variant genome, which measures 85% of the length of SV40-DNA. Each repeating unit consists of a segment of SV40-DNA derived from map positions 0.56–0.68, comprising about 65% of the repeating unit, and a segment derived from nonreiterated or less reiterated host cell DNA, making up about 35% of the repeating unit. The DNA of *ev*-1103 is made up of a repeating unit which is 8.8% of the length of SV40-DNA. In the major size-class of *ev*-1103 genomes (about 78% of the length of SV40-DNA) there are nine tandemly repeated segments. Each unit has a segment of SV40-DNA derived from the region around the *Hin*-A·C junction (about 0.65 map-units) and makes up about ¼–⅓ of the repeating unit. The rest of the DNA is derived from cell DNA, including reiterated and nonreiterated sequences. The topologic relationship of the different cell DNA sequences in *ev*-1103 is not entirely clear and is drawn in Fig. 11 with continuous reiterated and nonreiterated cell sequences.

In comparing the structure of the two variant genomes it is interesting that the SV40 segment of *ev*-1103 is included in the SV40 segment of *ev*-1101 (see below). However, the cell DNA present in *ev*-1101 does not correspond to that present in *ev*-1103. The latter findings suggest that the two variants probably arose by recombinations between SV40-DNA and cell DNA at different sites in the cell DNA. In this regard, Frenkel *et al.* (1974) recently characterized DNA from different series of uncloned substituted variants of SV40 by DNA–DNA hybridization and presented evidence for nonrandom differences in host sequences present. Concerning the SV40–cell DNA junctions in *ev*-1101 and *ev*-1103 DNA, at least one of the SV40 sites next to cell DNA is different in the two variants (the *Hin*-A·cell DNA junction). The *Hin*-C·cell DNA junctions, however, may be identical in the two variants (at 0.68 map-units); nucleotide sequence analyses will be required to settle this point. Obviously this result would have a bearing on the question of preferred sites on SV40-DNA for integration into cell DNA. In this connection it is interesting to point out that Khoury *et al.* (1975) have inferred from transcriptional analysis on SV40-transformed cells that *Hin*-C may contain a preferred site for integration.

Probably the most striking feature of the genomes analyzed is retention and repeti-

tion of that part of the SV40 genome which contains the initiation site for DNA replication (Fig. 11). This is reminiscent of the emergence of variants of coliphage Qβ-RNA described by Spiegelman and his co-workers (Mills *et al.*, 1973). In both instances, short polynucleotide segments evolve which retain the initiation site(s) for replication. In the case of *ev*-1103 only about 3% of the SV40 genome is retained, and this segment corresponds to the previously mapped initiation site. In all likelihood, retention and reiteration of this site have selective advantage during serial passage (see below).

Taking into account the structure of specific early passage variants described in the preceding paper (Brockman *et al.*, 1975) and those reported by Khoury *et al.* (1974) and by Mertz *et al.* (1974), we suggest that evolutionary variants arise by recombination, reiteration, and selection under conditions where helper viruses supply *trans* functions. SV40-infected cells have an active recombination system resulting in extensive recombination between and within SV40 genomes (Tai *et al.*, 1972; Lai and Nathans, 1974) and between SV40 and cellular DNA (Lavi and Winocour, 1972). Hence new genomes are formed containing deletions, duplications, rearrangements, and covalently linked cell DNA. Also, as a result of a reiteration process not presently understood, a relatively small segment of DNA can be enlarged to a size compatible with encapsidation (Davoli and Fareed, 1974). These various processes are illustrated in Fig. 12, which shows a hypothetical scheme for the generation of substituted variants. An alternative mechanism for amplifying viral DNA near the origin of replication has been suggested by Chow *et al.* (1974), Robberson and Fried (1974), and Folk (1974).

On the diverse pool of variant genomes generated by recombination, selective pressures must operate to increase the pool of those molecules that replicate faster and are large enough to be encapsidated and hence transmitted. The selective replicative advantage may be due simply to reiteration of the initiation site for viral DNA replication, since no other *cis* function for replication appears necessary (see below). As a consequence, early variants often contain duplicated initiation sites (Brockman and Nathans, 1974) and late variants contain many copies of the initiation site in each molecule and little SV40-DNA. However, most or perhaps all molecules with multiple initiation sites actually begin replication at only one of the possible sites (Fareed *et al.*, 1974; Brockman *et al.*, 1975; *ev*-1101 results). Although this could be due to a conformational change in the DNA following initiation, it could also be due to a limiting factor required for initiation (e.g., gene A product; Tegtmeyer (1972)) or perhaps a topological restriction. In either case the rate of initiation of a given DNA species would likely be proportional to the number of initiation sites per molecule.

The examination of replicating molecules of *ev*-1101 confirms the expectation that DNA replication begins in the SV40

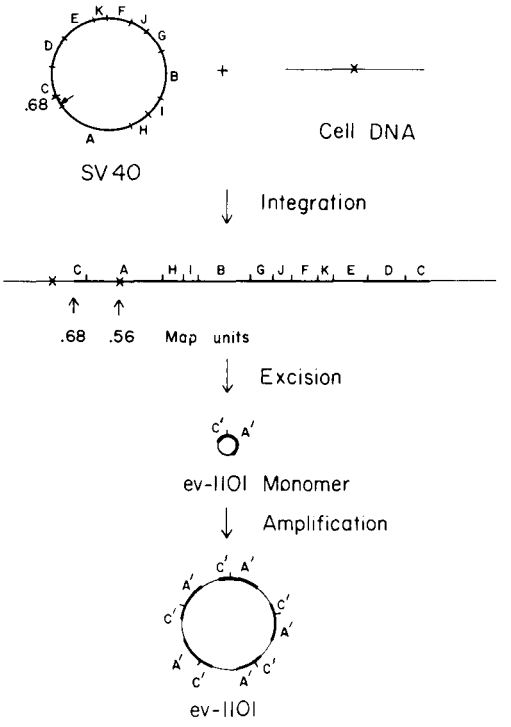

FIG. 12. A scheme for the generation of substituted variants with tandemly repeated segments of DNA. In the figure the formation of *ev*-1101 is illustrated.

segment near the *Hin*-A·C junction and terminates about half-way around the circular molecule. However, within the limits of sensitivity of the hybridization experiments reported in Table 2, neither *ev*-1101 nor *ev*-1103 DNA contain sequences derived from *Hin*-G where normal SV40-DNA replication terminates (Danna and Nathans, 1972). These data are thus compatible with our previous conclusion that termination of DNA replication is not specified by a nucleotide sequence (Brockman *et al.*, 1975). Further, the paucity of SV40-DNA sequences in *ev*-1103 (about 150 nucleotide pairs) makes it unlikely that this segment codes for a functioning initiator protein. Therefore, the initiation signal is probably the only *cis* function involved in SV40-DNA replication. (We should point out, however, that a contribution of the host sequences found in substituted variants has not been excluded.)

If the initiation signal is the only required *cis* function for replication, it follows that in the presence of infection by helper SV40 to supply *trans* functions, any double-stranded circular DNA molecule containing the SV40 signal will replicate. For example, it should be possible to construct a variety of replicatable DNA molecules by linking them to an SV40 initiation segment and then cloning in the presence of helper virus as was done for substituted variants. If these are of appropriate size, they will be encapsidated in SV40 coats; in this way potential transducing particles can be generated. Further, a plausible mechanism for SV40-stimulated cell DNA synthesis is suggested, namely, that cell DNA contains nucleotide sequences corresponding to the SV40 signal, either by chance and/or as a result of recombinational exchanges with SV40-DNA, leading to initiation at those sites.

One of the objectives of our studies of SV40 evolutionary variants has been to define the initiation signal for viral DNA replication. Clearly, molecules that retain this SV40 segment in functional form without any other viral DNA would be useful for nucleotide sequence analysis of the initiation site. We appear to be approaching that goal with *ev*-1103, which has about 150 nucleotide pairs from SV40; continued evolution appears to reduce this segment further (Gutai and Nathans, unpublished observations).

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